Author's response to reviews

Title: Quantitative proteomic analysis shows differentially expressed HSPB1 in glioblastoma as a discriminating short from long survival factor and NOVA1 as a differentiation factor between low-grade astrocytoma and oligodendroglioma.

Authors:

Marcela Gimenez (marcelagime@yahoo.com.br)
Suely K Marie (sknmarie@usp.br)
Sueli M Oba-Shinjo (suelimoba@usp.br)
Miyuki Uno (unomiyuki99@hotmail.com)
Clarice Izumi (cizumi@fmrp.usp.br)
João B Oliveira (bosco@genomika.com.br)
Jose C Rosa (jcrosa@fmrp.usp.br)

Version: 2
Date: 15 April 2015

Author's response to reviews: see over
We thank the reviewers for the criticism and suggestions that contributed to improve our article.

Reviewer: Donat Kögel

Major Compulsory Revisions
To this reviewer, it is unclear how/why the proteins shown in Table 1/Fig.1 were selected for further analysis. Were these really the only found proteins "known to participate in the process of tumor progression" or did the authors narrow their selection based on their previous research on these candidates?
Please explain. Along the same line, how does this selection relate to the model shown in Fig.5?
Proteins were selected as having a cut off frequency higher or lower expression than 1.5 times (log2 between 0.6 and -0.6) in relation to non neoplastic brain tissue (NN) and the statistical data p <0.05 between categories (non neoplastic, astrocytomas and oligodendrogliomas) explained in Table 1. The validation of proteomic data obtained by using isobaric tags was substantiated by the same findings obtained in other cohort, where NPM and RKIP were identified using other strategy in our laboratory (two dimensional gel electrophoresis (2D) and protein identification by mass spectrometry). Both proteins, NPM and RKIP, participate in two pathways RAF / MAP / ERK and PI3K / AKT / mTOR which have been described as related to tumor progression in glioma. Our present study unveiled that HSPB1, NUCL(or NCL), HSP90B and besides EGFR with differentially protein expressions in gliomas of different grade of malignancy, participate in the same pathway of NPM and RKIP according to MetaCore database (now Figure 6). Additional experiments are necessary to prove the activation of this pathway in the tumor progression, however, these results lay the groundwork for the comprehension of the signaling pathway involved in tumor progression. We modified the text, as highlighted in pages 14 and 15 (methods) and pages 20 to 22 (Network MetaCore analysis) to clarify these aspects.

Although there is a difference between the groups (Fig. 3 A and B), the considerable overlap makes it somewhat questionable whether HSP27 really would be truly useful in discriminating between good and bad prognosis.
A remarkable difference of HSP27 / HSPB1 protein expression level (p=0.00045) was observed when GBM cases of short (GBM-SS, 6±4 months, n=4) and long survival time (GBM-LS, 43±15 months, n=4) were compared and this finding was confirmed by immune-detection (IHC)(Figure 3D), and by western blot (Figure 3C) on a distinct, but still restricted, cohort of GBM cases. We also added figure 4A and B extended analysis of clinical significance of HSPB1 (ROC and Kaplan-Meier curves).

The discrimination between diffuse astrocytomas and oligodendrogliomas remains a diagnostic challenge and the new findings on NOVA1 expression are interesting. How do the authors reconcile their findings with the data of Zhi et al. (PLoS One. 2014 Oct;9(10):e109124.) who have shown that NOVA1 is expressed in astrocytoma of all grades with an increase in expression in relation to tumor grade?
In fact, we have also found NOVA1 expression in all grades of astrocytoma, as shown in the Figure below, with significant differential expression among the compared subgroups
(p=0.021, Kruskal Wallis’s test), and between non-neoplastic brain tissues (NN) and grade III astrocytoma (p<0.05, Dunn’s test), and NN and GBM (p<0.005, Dunn’s test).

Figure A: – Relative NOVA1 gene expression levels in astrocytomas of different grades compared to non-neoplastic brain tissues. Relative transcript levels of NOVA1 were determined in 23 non-neoplastic brain tissues (NN) and 150 astrocytomas, including 23 pilocytic astrocytomas -grade I (AGI), 26 low grade astrocytomas – grade II (AGII), 18 anaplastic astrocytomas – grade III (AGIII) and 83 glioblastomas, grade IV (AGIV). The relative expression levels of NOVA1 were analyzed by RT-qPCR using the SYBR Green approach. Quantitative data were normalized to the geometric mean of three reference genes suitable for the analysis: hypoxanthine phosphoribosyltransferase (HPRT), beta glucuronidase (GUSB) and TATA box binding protein (TBP). The equation $2^{-\Delta\Delta Ct}$ was applied to calculate the relative expression of NOVA1, where $\Delta\Delta Ct = [Ct$ of the target gene – geometric mean of the Ct of the reference genes] - mean CT of NN. The difference in NOVA1 expression level among the compared astrocytoma groups to NN group was statistically significant (Kruskal Wallis test, *p<0.021). A post-hoc Dunn’s test showed significant differences between NOVA1 expression on NN and AGIII (p<0.05), and NN and AGIV (p<0.005).

Our results of NOVA1 expression level in different grades of astrocytoma are similar to those presented by Zhi F et al, PlosOne 9(10): e109124. There are subtle differences between the two studies enumerated on the following table:

<table>
<thead>
<tr>
<th></th>
<th>Zhi F et al.</th>
<th>Our present study</th>
</tr>
</thead>
<tbody>
<tr>
<td>number of cases analyzed</td>
<td>90 cases (8 AGI, 26 AGII, 33 AGIII, 23 AGIV)</td>
<td>150 cases (23 AGI, 26 AGII, 18 AGIII, 83 AGIV)</td>
</tr>
<tr>
<td>reference genes used on RQ-PCR NOVA1 expression analysis</td>
<td>β-actin described on methodology, but GAPDH according to Figure 3F</td>
<td>hypoxanthine phosphoribosyltransferase (HPRT), beta glucuronidase (GUSB) and TATA box binding protein (TBP)¹</td>
</tr>
<tr>
<td>overall survival analysis by Kaplan-Meier</td>
<td>Figure 3H - Kaplan-Meier comparing cases presenting low and high NOVA1 expression, including all grades of astrocytomas showed significant</td>
<td>No significant difference of OS among GBM patients presenting low and high NOVA1 expression</td>
</tr>
</tbody>
</table>

1. TBP: TATA box binding protein
There is a major difference in the GBM cases included in our study compared to the study of Zhi F et al. (83 vs 23 cases), and it may be the reason of the difference in the median expression of NOVA1 in GBM cases. Additionally, we performed the relative expression analysis considering three reference genes. In our series of astrocytomas, the GAPDH expression was very variable and β-actin was very abundant, and both genes proved to be not suitable as references genes.1 We have not observed clinical impact on the overall survival time of NOVA1 differential expression among GBM cases. Astrocytomas of grade I and II present quite different and longer survival time compared of GBM patients, and therefore we have analyzed the Kaplan-Meier considering GBM patients only.

Astrocytomas of grade II to IV are considered diffusely infiltrative tumors, and it is difficult to obtain tumor surrounding brain tissue without tumor infiltration to be used as controls. For this reason, we have decided to use brain tissues collected from epilepsy surgery as non-neoplastic controls.

Although we have observed the increment of NOVA1 expression level with increase of malignancy among astrocytomas, the statistical differences were not significant, and as mentioned above no clinical impact was observed among GBM cases related to NOVA1 expression level. Therefore, we highlighted the other interesting finding of significant differential expression level of NOVA1 between low grade astrocytomas and oligodendrogliomas. And, we proposed that NOVA1 may be useful to discriminate these two types of low grade gliomas.

Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: Yes, but I do not feel adequately qualified to assess the statistics.
Declaration of competing interests:
I declare that I have no competing interests

Reviewer: Kai Stüler

For the evaluation of the manuscript the authors are asked to provide a more detailed scheme of the experimental set-up. Which samples have been pooled in 8-plex iTRAQ and what was the applied protein amount etc.? We added a supplementary figure S1 with a flow chart of sample preparation and added to the text the lacking information in material and methods section about quantities of tissue samples. Tumor samples from each patient were pooled, and quantified by the Bradford method (reference was added). Twenty five µg of each sample was added to a total of 100µg for each analyzed group, and it was labeled with a 8plex isobaric tag (iTRAQ).
Further the authors are asked to provide more demographic information about the analyzed patients as well as the genetic status about relevant prognostic markers of gliomas like e.g. IDH1 and MGMT status. Otherwise the authors will not consider relevant subgroups of glioma which will have an impact on the subsequent data interpretation and stratification. Data corresponding to age and survival time of the analyzed patients were added to the revised manuscript. IDH1 mutation was analyzed in this casuistry of gliomas and previously published\(^1\), as well as MGMT methylation status\(^2\). Both parameters were analyzed for GBM patients in association with HSPB1 expression level, as described below.

Another relevant issue is the selected criterion of 12 month and longer for long-term-survival. From the side of the reviewer it is questionable if the selected criterion is wisely chosen if the median survival time for GBM is 10 to 14 months. It is therefore suggested to adapt the criteria to the common sense of the scientific community and considering the 3-5% of patients surviving more than three years (Krex et al., 2007) for long-term surviving.

In response to the reviewer was added the average survival of patients analyzed and classified as short survival (GBM-SS, 6 +/- 4 months, n = 4) and long survival (GBM-LS, 43 +/- 15 months, n=4). For clarity and simplification intervals of <12 months for GBM-SS and >16 months for GBM-LS in the legend and axis of the figures were kept. We also corrected the figure 3 in its x axis which showed no intervals between short and long survivals and was in disagreement with our text on page 16 line 11. While reviewing the literature, we did not find a general consensus on the definition of long survival patients, but our main concern was to select samples that had a wide range between short and long survival, avoiding any overlap.

Further critical aspect is the application of the suggested proteins (HSPB1 and NOVA1) as diagnostic marker. At the moment the analysis based on the statistical evaluation relying of high patient numbers showing that different groups exist. But for diagnosis it is relevant to answer the question how reliable can a patient assign to a specific group by measuring the marker protein. Therefore, for evaluation of diagnostic performance statistic like e.g. ROC analysis have to be applied allowing to present sensitivity and specificity of the chosen marker. From the shown data insufficient performance can be concluded relying on a high false-positive or false-negative rate dependent of the selected thresholds. The author have to consider this aspect appropriately.

The HSPB1 expression levels of non-neoplastic brain tissues were compared to the expression levels of diffusely infiltrative astrocytomas (grades II to IV). Discrimination of variables was calculated by the receiver operator characteristic (ROC) curve utilizing area under curve and asymptotic significance. The continuous variables were categorized through a curve using ROC the value with the best sensitivity and specificity.


Figure B: ROC curves comparing HSPB1 expression levels of NN group compared to each grade of malignant astrocytomas, grade II to IV. The values of area, standard error under the nonparametric assumption, and asymptotic significance considering null hypothesis of true area - 0.5 are as follow:

<table>
<thead>
<tr>
<th></th>
<th>Area</th>
<th>Std error</th>
<th>Asymptotic Sig</th>
</tr>
</thead>
<tbody>
<tr>
<td>NN x AGII</td>
<td>0.544</td>
<td>0.085</td>
<td>0.605</td>
</tr>
<tr>
<td>NN x AGIII</td>
<td>0.732</td>
<td>0.081</td>
<td>0.012</td>
</tr>
<tr>
<td>NN x AGIV</td>
<td>0.901</td>
<td>0.030</td>
<td>0.000</td>
</tr>
</tbody>
</table>

The increasing value of the area in parallel to the increment of the malignancy, strongly suggests HSPB1 expression level as an indicator of tumor progression. The cut off value of 7.76 was determined based on the ROC curve to classify the GBM cases as presenting low or high HSPB1 expression. The Kaplan-Meier curve was calculated comparing GBM cases presenting ±3 fold this cut off value, i.e. GBM cases presenting HSPB1 expression level > 23.28 compared to those presenting HSPB1 expression level < 23.28. The resulted Kaplan=Meier curve with log rank of 0.007 is presented below.
Figure C: Kaplan-Meier curves. Overall survival time of GBM cases presenting HSPB1 expression level > 23.28 (3 fold of cut off value determined by ROC curve) (n=29) compared to GBM cases presenting HSPB1 expression level < 23.28 (n=48). Log rank = 0.007.

GBM patients presenting HSPB1 expression higher than 3 fold the cut off value calculated by ROC analysis (23.28) presented significant shorter overall survival time, and this results was independent to the IDH1 mutation status, according to multivariate proportional hazards analysis (Cox model):

<table>
<thead>
<tr>
<th>Variable</th>
<th>HR (95% CI)</th>
<th>p (value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSPB1 expression status(^1)</td>
<td>1.86 (1.14±3.03)</td>
<td>0.012</td>
</tr>
<tr>
<td>IDH1 mutation status(^2)</td>
<td>1.35 (0.64±2.84)</td>
<td>0.43</td>
</tr>
</tbody>
</table>

HR, hazard ratio; CI, confidence interval;
\(^1\)HSPB1 hyperexpressed compared to hypoexpressed
\(^2\)IDH1 mutated compared to wild type

Similar analysis was not feasible to MGMT methylation status, as such results were available for only 51 out of 83 GBM cases. Moreover, we have previously reported no impact of MGMT methylation status on the overall survival time among GBM cases (log rank, Mantel-Cox of 0.204) (Figure D).
Figure D: Kaplan-Meier curves. Overall survival time of GBM cases presenting methylated MGMT (n=23) compared to GBM cases presenting unmethylated MGMT (n=28). Log rank = 0.204.

The ROC curve analysis for HSPB1 expression for diffusely infiltrative astrocytomas (grade II to IV) and the Kaplan-Meier analysis were added into the text and also we added new Figure (4A and 4B) to illustrate these results.

Minor Essential Revisions
Figure 1 is unclear. Fold changes can only be calculated between and not within one group as presented in the figure (probably abundance?). The following text was added to legend of figure1: Proteins differentially expressed in astrocytomas and oligodendrogliomas. The protein expression is represented by Log2 fold change which is calculated dividing all the peaks by the average of the isobaric tag peak intensities appearing in the spectra included in NN category and that the spread shown for the log2 fold change of NN illustrates the variation of the isobaric tag peak intensities within the reference label in respect to their average.

In Figure 3g detailed data for the GBM short and GBM long survival is missing. Figure 3 was corrected and the differentiation bar of analysis of IHC between these two subjects, GBM short and long, was added.

Level of interest: An article of limited interest
Quality of written English: Acceptable
Statistical review: Yes, and I have assessed the statistics in my report.